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Molecular Cloning and Characterization of the Human Prostanoid DP Receptor*

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A cDNA encoding a functional human prostanoid DP (hDP) receptor has been constructed from a genomic clone and a fragment cloned by 3′-rapid amplification of cDNA ends-polymerase chain reaction. The hDP receptor consists of 359 amino acid residues with a predicted molecular mass of 40,276 and has the putative heptahelical transmembrane domains characteristic of G-protein-coupled receptors. The deduced amino acid sequence of the hDP receptor, when compared with all other members of the prostanoid receptor family, shows the highest degree of identity with the hIP and hEP_2 receptors, followed by the hEP_4 receptor. Radioreceptor binding studies using membranes prepared from mammalian COS-M6 cells transiently transfected with an expression vector containing the DP receptor cDNA showed that the rank order of affinities for prostaglandins and prostaglandin analogs, in competition for [^{3}H]prostaglandin D_2 (PGD_2) specific binding sites, was as predicted for the DP receptor, with $PGD_2 \gg PGE_2 > PGF_{2\alpha}$ = iloprost > U46619. The signal transduction pathway of the cloned hDP receptor was studied by transfecting the hDP expression vector into HEK 293(EBNA) cells. Activation of the hDP receptor with PGD_2 resulted in an elevation of intracellular cAMP and in mobilization of Ca^{2+}, but did not lead to generation of inositol 1,4,5-trisphosphate. Northern blot analysis of human tissues showed that the hDP receptor has a very discrete tissue distribution and was detectable only in retina and small intestine. In summary, we have cloned and expressed a functional cDNA for the hDP receptor.

Prostaglandin D_2 (PGD_2)[1] is formed in a variety of tissues including brain, spleen, lung, bone marrow, stomach, skin (1, 2), and also in mast cells. PGD_2 has been implicated in many physiological events both in the central nervous system and peripheral tissues. In the central nervous system, PGD_2 has been shown to affect the induction of sleep (3), body temperature (4), olfactory function (2 and references within), hormone release (2 and references within), and nociception (2 and references within). Since PGD_2 is the major prostanoid released from human mast cells upon immunological challenge (5), it is also considered to be an important mediator in allergic disorders such as allergic rhinitis (6). In addition, it has been tested as a potential intraocular pressure lowering agent for treatment of glaucoma (7, 8). Other roles for PGD_2, such as effects on platelet aggregation (1 and references within), systemic vasodilation, pulmonary constriction, and bronchoconstriction (1 and references within) appear, however, to be restricted to specific species. In addition, several of the physiological effects assigned to PGD_2, including bronchoconstriction, may be attributable to the cross-reactivity of PGD_2 with the prostaglandin $F_{2\alpha}$ and/or thromboxane A_2 receptors (9).

The physiological and pathophysiological actions of PGD_2 are mediated through interaction with the prostanoid DP receptor.[2] Specific binding sites for the DP receptor have been studied using membranes from human platelets (10), rat brain (11), human basophils (12), and mouse mastocytoma P-815 cells (13). Activation of the DP receptor has been shown to result in an increase in both intracellular cAMP ($[cAMP]_i$) (14, 15) and Ca^{2+} ($[Ca^{2+}]_i$) (16), and the receptor is potentially regulated by phosphorylation events (13). DP receptors are thought to be localized in platelets (17 and references within), neutrophils (18–20), non-chromaffin cells from adrenal medulla (16), and smooth muscle cells from several tissues and nervous tissue, including the central nervous system (17). However, the DP receptor is the least abundant of the prostanoid receptors and is, therefore, the least well characterized.

The study of the human prostanoid receptors is rapidly advancing with the recent cloning of the human (h) thromboxane A_2 (TP) (21), prostaglandin $F_{2\alpha}$ (FP) (22, 23), and prostacyclin (IP) (24–26) receptors and four distinct subtypes of the prostaglandin E_2 (EP_1 (27), EP_2 (28), EP_3 (29–31), and EP_4 (32, 33)[3]) receptor. Here we report the cloning of the human prostaglandin D_2 (DP) receptor, and the nucleotide and deduced amino acid sequences are described. The radioligand binding and functional characteristics of the cloned and expressed hDP receptor have been addressed, in addition to its tissue distribution.

MATERIALS AND METHODS

Human Genomic Library Screening—In order to clone the hDP prostanoid receptor, a mouse DP receptor DNA probe was first generated using the polymerase chain reaction as described below. Two oligonucleotides based on the mouse DP receptor amino acid sequence (34)

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U31332, U31098, and U31099.

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[1] The abbreviations used are: PG, prostaglandin; bp, base pair(s); kb, kilobase(s); TMD, transmembrane domain; RACE, rapid amplification cDNA ends; PCR, polymerase chain reaction; GPCR, G-protein-coupled receptors; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

[2] Prostanoid receptors are designated following the recommendation of the IUPHAR Commission on Receptor Nomenclature and Classification (52).

[3] The human prostaglandin E_2 (PGE_2) receptor described by An *et al.* (33) and Bastien *et al.* (32) as the EP_2 subtype has now been reclassified as the EP_4 subtype since it has negligible affinity (K_i = 23 μM) for the EP_2-selective agonist butaprost. In contrast, the PGE_2 receptor described by Regan *et al.* (28) displays moderate affinity (K_i = 1.4 μM) for butaprost and has therefore been designated as the EP_2 subtype.

were designed and synthesized (Research Genetics, Cambridge, MA). The 5'-sense 128-fold degenerate oligonucleotide (5'-ATG AA(T,C) GA(G,A) (T,A)(G,C)I TA(T,C) (A,C)GI TG(T,C) CA-3') was based on the first eight amino acids (MNESYRCQ), and the 3'-antisense 16-fold degenerate oligonucleotide (5'-AA (G,A)CA CCA IGT ICC IGG (G,A)CA (G,A)TA (T,C)TG-3') was based on nine amino acids (QYCPGTWCF) which occur in the second extracellular loop of the mouse DP receptor. The polymerase chain reaction protocol for the amplification, performed on a DNA thermal cycler 480 (Perkin Elmer, Montreal, Canada) using *Taq* polymerase (Boehringer Mannheim, Laval, Quebec) and 1.5 μg of mouse genomic DNA (Clontech) as template, was as follows: denaturation at 95 °C for 60 s, annealing at 52 °C for 30 s, and extension at 72 °C for 40 s, for 35 cycles. The 549-bp amplified product was subsequently isolated from a low-melt agarose gel, random prime labeled (T7 Quick Prime Kit, Pharmacia, Baie D'urfe, Quebec) with [^{32}P]dCTP (DuPont NEN, Mississauga, Ontario), and added to a 50% formamide hybridization buffer to screen 1.2 × 10^6 plaques from a λEMBL3 T7/SP6 *Sau*3AI human genomic library (Clontech). Two positive phage clones, λhDPgc.1 and λhDPgc.2, were plaque-purified, and DNA was prepared by the plate lysate method (35).

Restriction mapping of λhDPgc.1 and λhDPgc.2 DNA was performed using *Xho*I, *Sac*I, *Sfi*I, *Eco*RI, *Xba*I, *Bam*HI, *Hin*dIII, and *Asp*718I. Restriction digest analysis showed that both clones were identical. Southern blot analysis of the digested DNA was performed with either the ^{32}P-labeled mouse DP genomic DNA fragment or a 29-mer 4-fold degenerate oligonucleotide, hDP-VII(+) (5'-ATIGTIGA(T,C)CCITG-GATITT(T,C)ATIATITT-3'), based upon 10 amino acids from transmembrane domain (TMD) VII (IVDPWIFIIF) from the mouse DP receptor (34). Results from the Southern analysis identified a 3-kb *Eco*RI fragment which hybridized with the 549-bp mouse DP DNA probe but not the oligonucleotide probe and, in addition, a 4.5-kb *Xho*I/*Eco*RI fragment originating from the 3'-end of the genomic insert which hybridized with the hDP-VII(+) oligonucleotide probe but not the mouse DP DNA probe. These fragments were isolated and subcloned into Bluescript pKS vectors (Stratagene) for sequence analysis using an ABI 373 Stretch automated sequencer. The first 1.5 kb of the 3-kb *Eco*RI fragment was sequenced on both strands using KS and SK primers or primers generated from the determined sequences. This sequence was found to contain the 5' end of the coding region up to and including an exon/intron splice site located near the end of TMD VI as well as part of the intron. The 3' end of the 4.5-kb *Xho*I/*Eco*RI fragment was sequenced as above and found to contain the continuation of the coding region from the end of TMD VI through to the end of TMD VII, which was also the end of the genomic clone.

3'-RACE-PCR Cloning—To obtain the missing 3'-coding region of the hDP receptor, 3'-RACE-PCR was performed on human small intestine, lung, thymus, kidney, and uterus cDNA libraries (Clontech). Anchored primers, complementary to λgt10 or λgt11 DNA sequences at their multiple cloning site junctions, and sense primers, AP-1 (5'-CCTGGC-CATGGCACTGGAGTGCTGG-3') (end of TMD III), AP-2 (5'-GCTGTG-CAACCTCGGCGCCATGCGC-3'] (end of TMD V), and AP-3 (5'-CTC-CGAGCCTTGCGATTTCTATCTG-3') (beginning of TMD VII), based on the sequence obtained from the hDP receptor genomic clone were either purchased from Clontech or synthesized as above. The polymerase chain reaction protocol for all rounds of amplification was as follows: denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s, for 35 cycles. For the first reaction, 2 μl (~10^7 plaque-forming units/μl) of the cDNA library was amplified using a λgt primer and AP-1. For the second amplification, 1/50 (2 μl) of the first polymerase chain reaction was reamplified with the same λgt primer and either AP-2 or AP-3. Amplified products were then analyzed by Southern blotting according to standard protocols (35) using the ^{32}P-labeled oligonucleotide hDP-VII(+). Hybridizing polymerase chain reaction fragments were subsequently cloned into Bluescript vector pKS and sequenced as above. An amplified fragment (510 bp) from the small intestine cDNA library was found to extend an additional 157 bp (including an in-frame putative stop codon) as compared with the sequence at the 3' end of the hDP genomic clone. Shorter amplified fragments of similar size were also obtained from the lung and uterus cDNA libraries. The lung fragment contained only 53 bp of additional sequence information and did not extend to the putative stop codon.

Construction of a cDNA Encoding the Human DP Receptor—The following strategy was used in order to construct a cDNA containing the full-length coding region for hDP: the 5' end from the 3-kb *Eco*RI genomic fragment in pKS was digested with *Sma*I and *Pst*I releasing a 0.75-kb fragment which encoded amino acid residues 1 to 232 of the hDP receptor. The 0.75-kb fragment was then ligated into the pKS construct, previously digested with *Sma*I and *Pst*I, which contained the small intestine 3'-RACE-PCR fragment encoding amino acid residues 233 to 359. This construct was designated pKS-hDP.

Construction of pcDNA3-hDP and pCEP4-hDP Mammalian Expression Vectors—Digestion of pKS-hDP with *Not*I and *Xho*I released the hDP 1.2-kb cDNA which was subcloned into the same restriction sites of both the pcDNA3 and pCEP4 mammalian expression vectors (Invitrogen). The correct orientation was verified by *Bgl*II digestion. These vectors, termed pcDNA3-hDP and pCEP4-hDP, were subsequently used in transfection experiments for receptor binding and signal transduction assays, respectively.

Northern Blot Analysis—Poly(A$^+$) RNA samples (3 μg) from 19 different human tissues (Clontech) were subjected to electrophoresis in a 1.2% agarose gel containing 0.22 M formaldehyde and were then transferred to a nylon membrane (Nytran$^+$, Schleicher and Schuell). The nylon membrane was hybridized at 42 °C overnight in 50% formamide, 4 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 100 mg/ml sonicated salmon sperm DNA containing 1 × 10^6 cpm/ml of a ^{32}P-random-prime-labeled 0.9-kb *Sma*I hDP fragment originating from the 3-kb *Eco*RI genomic fragment. The nylon membrane was washed with 0.1 × SSC containing 0.1% SDS at 55 °C and then exposed at −80 °C for 14 days with two intensifying screens.

pcDNA3-hDP Expression in COS-M6 Cells and [^{3}H]PGD$_2$ Binding Assays—The pcDNA3-hDP vector was transfected into COS-M6 cells by cationic liposome-mediated transfer using LipofectAMINE™ reagent (36) (Life Technologies, Inc./BRL, Burlington, Ontario). The cells were maintained in culture for 48 h, harvested, and subjected to lysis by nitrogen cavitation (37), and membranes were prepared by differential centrifugation (1000 × *g* for 10 min, then 100,000 × *g* for 30 min). [^{3}H]PGD$_2$ binding assays were performed in 10 mM BES/KOH (pH 7.0) containing 1 mM EDTA, 10 mM $MnCl_2$, 0.8 nM [^{3}H]PGD$_2$ (115 Ci/mmol; DuPont NEN), and 60 μg of protein from the 100,000 × *g* membrane fraction. Incubations were conducted for 60 min at 30 °C prior to separation of the bound and free radioligand by rapid filtration through GF/B filters presoaked in 10 mM BES/KOH (pH 7.0) containing 0.01% (w/v) bovine serum albumin. Filters were washed with 16 ml of soaking buffer, and the residual [^{3}H]PGD$_2$ bound to the filter was determined by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding which was determined in the presence of 1 μM PGD$_2$.

pCEP4-DP Expression in HEK 293(EBNA) Cells—Stable expression of the hDP receptor was achieved by transfection of the pCEP4-hDP plasmid into HEK 293(EBNA) cells (maintained under selection with GENETICIN® (G418) (Life Technologies, Inc.)), using LipofectAMINE® reagent as described above. Cells were maintained in culture for 48 h post-transfection and then grown in the presence of 200 μg/ml hygromycin B (Calbiochem) for 3–4 weeks to select for resistant cells expressing the hDP receptor. These cells were subsequently used for signal transduction assays.

cAMP Assays in HEK 293(EBNA) Cells Expressing the Human DP Receptor—hDP-HEK 293(EBNA) cells were maintained in culture in Dulbecco's modified Eagle's medium growth medium (Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 20 units/ml penicillin G, 20 μg/ml streptomycin sulfate, 250 μg/ml G418, and 200 μg/ml hygromycin B). The medium was replaced 16–24 h prior to harvesting the cells (at approximately 80% confluence). The cells were harvested by incubation in enzyme-free cell dissociation buffer (Life Technologies, Inc./BRL, Burlington, Ontario), washed with HEPES-buffered Krebs-Ringer solution (1.25 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM KCl, 124 mM NaCl, 8 mM glucose, 1.25 mM KH_2PO_4, and 25 mM HEPES/KOH (pH 7.4)) and then resuspended in the same buffer. The cAMP assay was performed in a final volume of 0.2 ml of HEPES-buffer Krebs-Ringer solution containing a 100 μM concentration of the phosphodiesterase type IV inhibitor RO-20–1724 (BIOMOL Research Laboratories, Plymouth Meeting, PA) to prevent hydrolysis of cAMP. RO-20–1724 and the compounds under evaluation were added to the incubation mixture in dimethyl sulfoxide (Me_2SO) or ethanol to a final vehicle concentration, 0.3% (v/v), which was kept constant in all samples. The reaction was initiated by the addition of 1.5 × 10^5 cells per incubation. Samples were incubated at 37 °C for 15 min, and the reaction was then terminated by immersing the samples in boiling water for 3 min. Cell viability was always ≥96% as determined by trypan blue exclusion. Measurement of cAMP was performed by radioimmunoassay using ^{125}I-cAMP (Amersham, Oakville, Ontario).

Intracellular Calcium Assays in HEK 293(EBNA) Cells Expressing the Human DP Receptor—Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements were performed using the hDP-HEK 293(EBNA) stable cell line. The cells were harvested with enzyme-free cell dissociation buffer, recov-

ered by centrifugation at 300 × *g* for 5 min at 22 °C, and resuspended at 2 × 10^6 cells/ml in Dulbecco's modified Eagle's medium growth medium without G418 or hygromycin B. The cells were incubated at 37 °C, 6% CO_2 for 45 min with 2 μM fura-2/AM (Molecular Probes). Unincorporated fura-2/AM was removed by three washes in Hanks' buffered saline solution (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.9 mM $MgCl_2$, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose) followed by centrifugation at 300 × *g* for 5 min at 22 °C. The cells were then resuspended at a final concentration of 10^6 cells/ml in Hanks' buffered saline solution. $[Ca^{2+}]_i$ was measured following an agonist challenge of 2 × 10^6 fura-2/AM loaded cells using a Perkin-Elmer LS-5 spectrophotometer essentially as described previously (38). Cells were viable at the end of each experiment, as monitored by the large increase in $[Ca^{2+}]_i$ observed upon the addition of a 1 μM concentration of the calcium ionophore ionomycin (Sigma) followed by quenching of the $[Ca^{2+}]_i$ response with 1 mM EDTA.

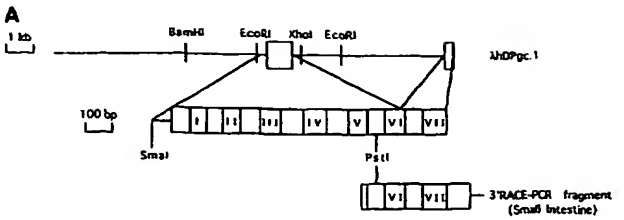
Inositol 1,4,5-Trisphosphate Assays in HEK 293(EBNA) Cells Expressing the Human DP Receptor—Inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) measurements were performed using the hDP-HEK 293(EBNA) and hEP_1-HEK 293(EBNA) stable cell lines and control HEK 293(EBNA) cells. The cells were harvested by incubation in enzyme-free cell dissociation buffer, washed with Hanks' buffered saline solution, and then resuspended in the same buffer. The $Ins(1,4,5)P_3$ assay was performed in a final volume of 0.4 ml of Hanks' buffered saline solution containing 10 mM LiCl. The compounds under evaluation were added to the incubation mixture in Me_2SO_4 to a final concentration of 0.5% (v/v), which was kept constant in all samples. The reaction was started with the addition of 5 × 10^5 cells per incubation. Samples were incubated at 37 °C for 10 min, and the reaction was terminated by the addition of 20% ice-cold trichloroacetic acid. $Ins(1,4,5)P_3$ was extracted and measured by $[^3H]Ins(1,4,5)P_3$ radioreceptor assay (DuPont NEN) according to the manufacturer's instructions except that the bound and free radioligand was separated by rapid filtration through GF/C filters presoaked in 10 mM HEPES/KOH (pH 8.0). Filters were washed with 4 ml of 10 mM HEPES/KOH (pH 8.0), and the residual $[^3H]Ins(1,4,5)P_3$ bound to the filter was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Cloning and Construction of a cDNA Coding for the Human DP Receptor—In order to clone the hDP receptor, a mouse DP receptor fragment was first amplified from mouse genomic DNA using the polymerase chain reaction as described under "Materials and Methods." This mouse DP DNA probe was subsequently used to screen a human genomic library, and a hDP genomic clone was identified. The clone was, however, missing coding information from the carboxyl-terminal end of the receptor. The additional coding sequence was derived by 3'-RACE-PCR of a human small intestine cDNA library which made it possible to construct the entire coding region for the hDP receptor from the hDP gene and the 3'-RACE-PCR fragment (Fig. 1*A*). The hDP genomic clone was found to contain an intron with consensus donor and acceptor splice site sequences (39) near the end of TMD VI (Fig. 1*B*). This position is identical with that found in the mouse DP gene (Hirada) and homologous in position to that of the hTP receptor gene (40) as well as other members of the human prostanoid receptor gene family.[4] This suggests that the prostanoid receptor family arose from a single ancestral gene.

The hDP cDNA contains a 1,080-bp open reading frame coding for a 359-amino-acid protein (Fig. 1*B*) with a calculated molecular mass of 40,276. The ATG assigned as the initiator codon matches reasonably well to the Kozak consensus sequence (4/5 nucleotides) for translation initiation (41). The genomic clone also contains an in-frame TAG stop codon 171 bp upstream of the predicted start codon (Fig. 1*B*). The hDP protein contains three potential *N*-glycosylation sites, Asn-10, Asn-90, and Asn-297, in the NH_2 terminus and the first and third extracellular loops, respectively. There are also two potential protein kinase C phosphorylation sites, Ser-50 and Thr-

[4] Y. Boie and M. Abramovitz, unpublished observations.



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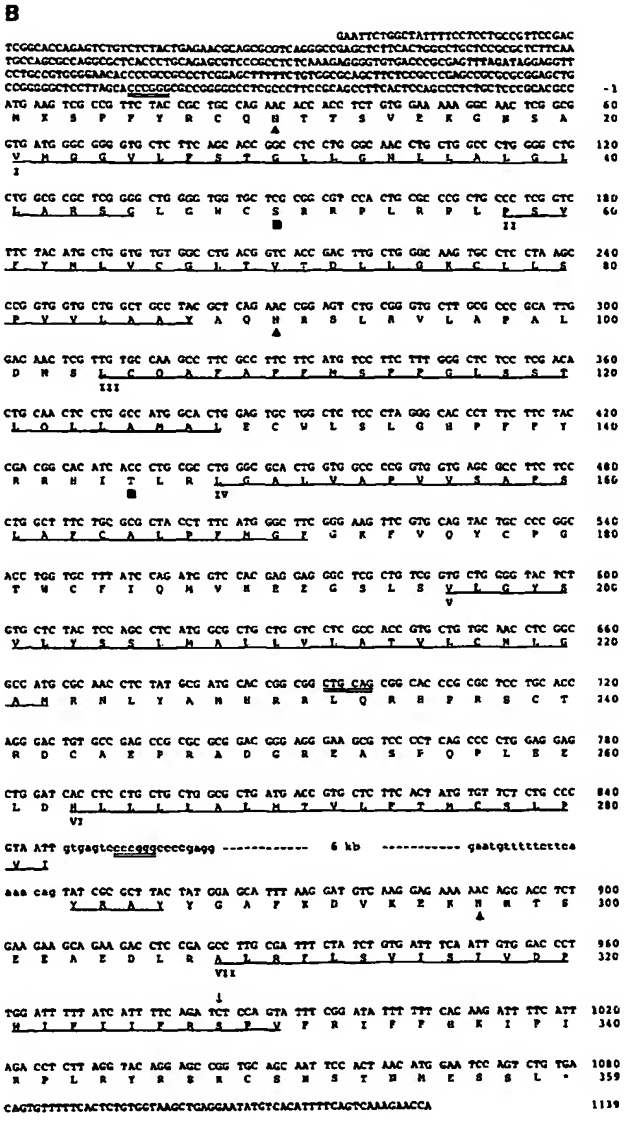

FIG. 1. **Nucleotide and deduced amino acid sequence of the human prostanoid DP receptor.** *A*, the relationship between the genomic clone, λhDPgc.1, and the 3'-RACE-PCR product derived from the small intestine cDNA library is shown. The coding regions in λhDPgc.1 are indicated by *open boxes* and expanded directly below with the intron removed and the seven putative transmembrane domains numbered in *Roman numerals*. The full-length coding region of the hDP receptor was obtained by joining the *SmaI*/*PstI* genomic fragment to the 3'-RACE-PCR fragment at the common *PstI* site (see "Materials and Methods" for details). *B*, the nucleotide and deduced amino acid sequences of the human DP receptor gene. The nucleotides and amino acids, shown below, are *numbered* according to the initiator methionine. The end of the genomic clone is indicated by an *arrow* above the last nucleotide. The upstream in-frame stop codon, TAG, at position −171, is in *bold*, and an *asterisk* denotes the position of the translation stop codon. The nucleotides in the intron are in *small letters*. The positions of the putative transmembrane segments I-VII (based on the hydropathicity profile) are underlined. *N*-Glycosylation sites (▲) and poten-

145, located in the putative first and second cytoplasmic loops, respectively. In addition, there are six more potential phosphorylation sites in the carboxyl-terminal tail, comprised of five serine residues and one threonine residue which may be important in receptor desensitization (42). Hydropathy analysis (43) of the deduced amino acid sequence of the hDP receptor confirmed the presence of the seven putative TMDs which are characteristic of G-protein-coupled receptors (GPCR) (44). The hDP receptor also shares limited, but significant, amino acid identity with members of the rhodopsin-like family within the GPCR superfamily (44).

Expression of the Human DP Receptor in COS-M6 Cells—[^{3}H]PGD_2 binding assays were performed using membranes prepared from transient transfection of COS-M6 cells with pcDNA3-hDP. Preliminary receptor binding assays, performed to establish assay conditions, demonstrated that [^{3}H]PGD_2 specific binding was optimal at pH 7.0 and was maximally stimulated in the presence of 10 mM Mn^{2+}. In addition, Scatchard analysis showed that [^{3}H]PGD_2 specific binding to the hDP receptor was of high affinity and conformed to a one-site model (Accufit Two-Site saturation software, Beckman Instruments) with an equilibrium dissociation constant (K_D) of 1.5 nM (Fig. 2*A*). [^{3}H]PGD_2 specific binding was saturable, with a maximum number of specific binding sites (B_{max}) of approximately 1.2 pmol/mg of membrane protein. Scatchard analysis was also performed in the presence of the slowly hydrolyzable GTP analog GTPγS (100 μM) (Fig. 2*A*). In this case, the affinity of [^{3}H]PGD_2 for hDP was reduced approximately 20-fold (K_D = 28 nM) demonstrating that dissociation of the associated G-proteins from the hDP receptor converts the receptor to a low affinity state with respect to ligand binding, although the B_{max} (1.3 pmol/mg of protein) was unchanged.

Equilibrium competition binding assays were conducted in order to determine the relative affinities of prostaglandins and related synthetic analogs for the hDP receptor (Fig. 2*B*). The most effective competing ligand was PGD_2 which displayed a K_i value of 1.1 nM. The selective DP agonist BW 245C (45) and the selective DP antagonist BW 868C (46) were equipotent with K_i values of 0.9 nM and 1.7 nM, respectively. PGE_2 was approximately 100-fold less effective as a competing ligand with a K_i of 101 nM, while $PGF_{2\alpha}$, the IP agonist iloprost, and the thromboxane A_2 mimetic U46619 were less active in competition for [^{3}H]PGD_2 specific binding to the hDP receptor. The rank order of affinities for prostaglandins and related synthetic analogs at the hDP receptor was therefore: BW 245C = PGD_2 = BW 868C ≫ PGE_2 > $PGF_{2\alpha}$ = iloprost > U46619, as predicted for the DP receptor from pharmacological studies (47, 48). There was no detectable [^{3}H]PGD_2 specific binding observed using COS-M6 cell membranes from mock-transfected cells under these experimental conditions.

Increases in cAMP Production in HEK 293(EBNA) Cells Expressing the Human DP Receptor—$[cAMP]_i$ production was increased in HEK 293(EBNA) cells stably expressing the hDP receptor following challenge with prostaglandins and related synthetic analogs (Fig. 3). The DP-selective agonist BW 245C was the most potent compound to increase cAMP production with an EC_{50} value of 0.7 nM. In comparison, PGD_2 was 8-fold less potent with an EC_{50} value of 6 nM. The putative DP-selective antagonist BW A868C marginally increased cAMP, producing 20 pmol of cAMP/1.5 × 10^5 cells *versus* 140 pmol of cAMP/1.5 × 10^5 cells for PGD_2 at concentrations of 1 μM. Finally, PGE_2, PGE_1, $PGF_{2\alpha}$, iloprost, and U46619 all had EC_{50} values of >500 nM. The rank order of potency of prostaglandins and related synthetic analogs was: BW 245C > PGD_2 ≫ PGE_2 > PGE_1 > $PGF_{2\alpha}$ > iloprost ≈ U46619. This rank order of potency has been predicted for the DP receptor from previous pharmacological studies (17). The increase in cAMP production upon agonist stimulation of hDP-HEK 293(EBNA) cells is mediated through the hDP receptor since these responses did not occur in comparable experiments performed in control HEK 293(EBNA) cells. BW 245C, PGD_2, BW A868C, $PGF_{2\alpha}$, iloprost, and U46619 did not increase cAMP production in control cells

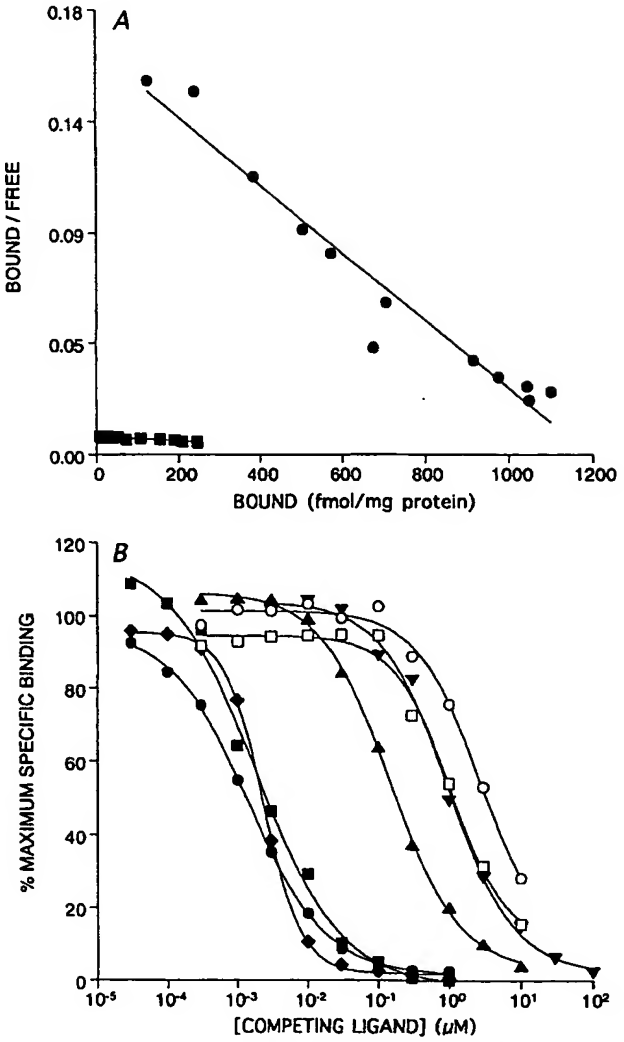

FIG. 2. **Competition for [^{3}H]PGD_2 specific binding to the human DP receptor expressed in COS-M6 cells.** Radioligand membrane binding assays were conducted as described under "Materials and Methods." *A*, Scatchard analysis of [^{3}H]PGD_2 specific binding to membranes from COS-M6 cells expressing hDP. Receptor binding assays were performed over a concentration range of 0.4–10 nM [^{3}H]PGD_2 in the presence (■) and absence (●) of 100 μM GTPγS. At each radioligand concentration, total and nonspecific binding was determined in the absence and presence of 1 μM PGD_2. The deduced specific binding saturation isotherm was obtained by subtracting nonspecific from total binding, and linear transformation was performed according to the method of Scatchard using Accufit Two-Site saturation software (Beckman Instruments). *B*, equilibrium competition binding assays were conducted in the presence of 0.03–1000 nM BW 245C (●), PGD_2 (■), and BW A868C (♦) and 0.3 nM–100 μM PGE_2 (▲), $PGF_{2\alpha}$ (▼), iloprost (□), and U46619 (○). BW 245C and BW A868C were generous gifts from The Wellcome Foundation Ltd. Data points are the mean of duplicate experimental values and are representative data from two experiments giving similar results.

tial protein kinase C phosphorylation sites (■) are indicated below the amino acids. The two *SmaI* and one *PstI* restriction sites are *double-underlined*.

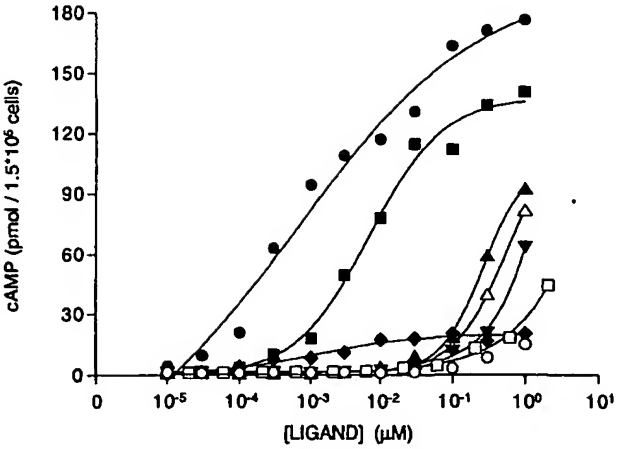

FIG. 3. **Increases in cAMP in HEK 293(EBNA) cells expressing the human DP receptor.** DP-HEK 293(EBNA) cells were incubated in HEPES-buffered Krebs-Ringer buffer and 100 μM RO-20–1724 as described under "Materials and Methods." The incubation medium also contained, as shown in this figure: BW 245C (●), PGD_2 (■), BW 868C (♦), PGE_2 (▲), PGE_1 (Δ), $PGF_{2\alpha}$ (▼), Iloprost (□), and U46619 (○) over a concentration range up to 1 μM. The reaction was initiated by addition of 1.5×10^5 cells, and the samples were incubated at 37 °C for 15 min prior to termination of the reaction by immersing the samples in boiling water for 3 min. Measurement of cAMP in the samples was performed by radioimmunoassay. Results have been expressed as picomoles of cAMP produced per 1.5×10^5 cells at each ligand concentration. Data points are the mean of duplicate experimental values and are representative data from two experiments giving similar results.

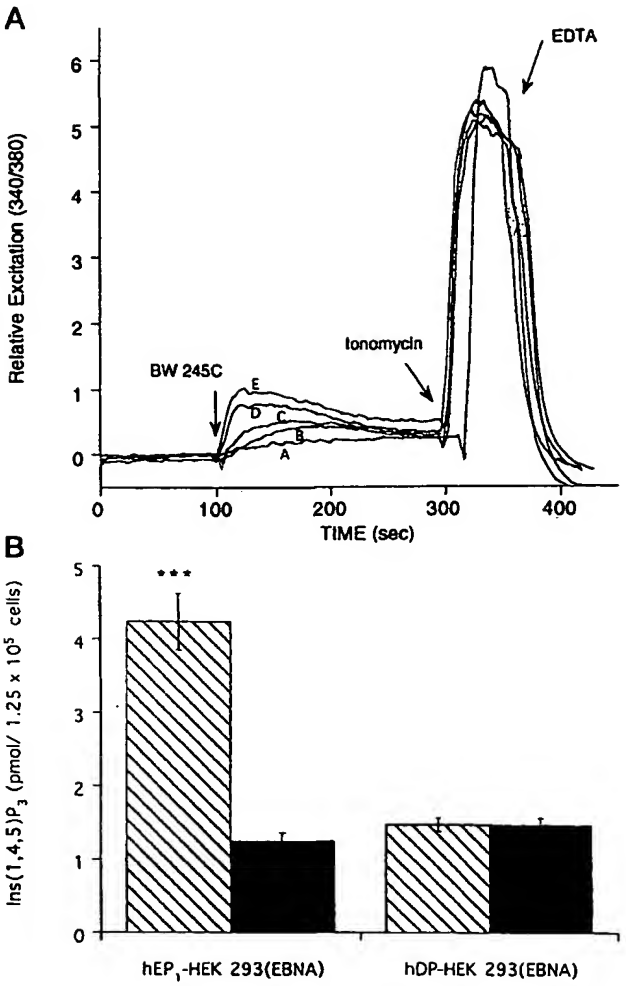

FIG. 4. *A*, increases in $[Ca^{2+}]_i$ in fura-2/AM loaded HEK 293(EBNA) cells expressing the DP receptor. hDP-HEK 293(EBNA) cells were harvested and prepared as detailed under "Materials and Methods." hDP-HEK 293(EBNA) cells (2×10^6) were then challenged with vehicle (Me_2SO_4 at 0.2% (v/v) final) (*A*) or 1.0 nM (*B*), 10 nM (*C*), 100 nM (*D*), and 1000 nM (*E*) BW 245C. Similar results were also obtained with PGD_2 over the same concentration range (data not shown). At the end of each experiment, cells were challenged with 1 μM ionomycin to release $[Ca^{2+}]_i$ followed by quenching of the $[Ca^{2+}]_i$ released with 1 mM EDTA. These are representative data from two experimental observations giving similar results. The relative excitation ratio of 340/380 has been adjusted to 0 after the establishment of the baseline at time = 80 s. *B*, $Ins(1,4,5)P_3$ measurements in hDP-HEK 293(EBNA) and hEP_1-HEK 293(EBNA) cells. The cells were harvested and prepared as detailed under "Materials and Methods." hEP_1-HEK 293(EBNA) and hDP-HEK 293(EBNA) cells (5×10^5) were then challenged with either 300 nM PGE_2 or 1 μM PGD_2, respectively (*hatched bars*), or with vehicle (Me_2SO_4 at 0.5% v/v final) (*solid bars*). The reaction was terminated by the addition of 20% (v/v) ice-cold trichloroacetic acid, and $Ins(1,4,5)P_3$ was measured as detailed under "Materials and Methods." Results have been expressed as picomoles of $Ins(1,4,5)P_3$ per 1.25×10^5 cells for each cell type and ligand concentration. Data points are the mean ± S.E. of triplicate experimental values and are representative data from three experiments giving similar results.

when tested at concentrations up to 1 μM (data not shown). In contrast, PGE_2 and PGE_1 increased cAMP production in HEK 293(EBNA) cells in a concentration-dependent manner. The magnitude of these responses, however, was small (6 pmol of cAMP/1.5×10^5 cells), approximately 20-fold lower than the responses for these ligands obtained in HEK 293(EBNA) cells expressing the DP receptor. These data suggest that HEK 293(EBNA) cells contain a small population of endogenous EP receptor(s) capable of responding to PGE_2 and PGE_1. In summary, the hDP receptor signals through coupling to elevation in $[cAMP]_i$ production.

Increases in $[Ca^{2+}]_i$ in Fura-2/AM Loaded HEK 293(EBNA) Cells Expressing the hDP Receptor—Challenge of fura-2/AM loaded HEK 293(EBNA) cells expressing the hDP receptor with 1–1000 nM PGD_2 or BW 245C increased $[Ca^{2+}]_i$ mobilization in a concentration-dependent manner (Fig. 4*A*). In addition, there was no change in $[Ca^{2+}]_i$ when the cells were challenged with the vehicle (Me_2SO_4 at 0.2% v/v). The addition of 1000 nM PGD_2 or BW 245C to control HEK 293(EBNA) cells did not provoke any change in $[Ca^{2+}]_i$ (data not shown) which demonstrates that increases in $[Ca^{2+}]_i$ after agonist challenge in hDP-HEK 293(EBNA) cells is mediated through the hDP receptor. However, challenge of the fura-2/AM loaded hDP-HEK 293(EBNA) cells with forskolin to activate directly adenylyl cyclase also evoked a $[Ca^{2+}]_i$ response (data not shown) which was similar to that achieved with PGD_2. This also occurred in control HEK 293(EBNA) cells which did not express the DP receptor. These data suggest that the changes in $[Ca^{2+}]_i$ in the DP-HEK 293 (EBNA) cells may be provoked through increases in $[cAMP]_i$ and not through DP receptor activation of the phospholipase C/inositol phosphate generation pathway. This was investigated further by measuring $Ins(1,4,5)P_3$ generation following activation of the DP receptor. Challenge of DP-HEK 293(EBNA) cells with 1 μM PGD_2 did not increase the level of $Ins(1,4,5)P_3$ above the basal level observed in the absence of agonist (Fig. 4*B*). Similar results were obtained using control HEK 293(EBNA) cells. In contrast, challenge of hEP_1-HEK 293(EBNA) cells with 300 nM PGE_2 resulted in a 4-fold increase in $Ins(1,4,5)P_3$ when compared with the basal level ($p < 0.001$). The signal transduction pathway of the hEP_1 receptor is known to result in activation of phospholipase C and the subsequent generation of $Ins(1,4,5)P_3$. The DP receptor, however, does not appear to signal through this pathway. This is supported by studies showing that PGD_2 can cause a cAMP-dependent Ca^{2+} influx in non-chromaffin cells from bovine adrenal medulla

(16). The precise mechanism through which the hDP receptor mediates the effect on calcium mobilization in HEK 293(EBNA) cells is, therefore, being characterized in more detail.

Northern Blot Analysis—Northern blot analysis was performed on poly(A)$^+$ RNA from 19 different human tissues. Hybridizing bands were detected only in the small intestine and retina (Fig. 5). Three different size transcripts were detected in the retina which may result from the use of different polyadenylation signals in the 3′-untranslated region. The inability of the probe to detect DP message in the other tissues supports previous data in the literature (47) and suggests that expression of the receptor may be limited and, in addition, may be restricted to specific cell types within tissues. In the brain, for example, PGD_2 is the most potent sleep-promoting prostanoid known (4), and the sleep-promoting zone has been localized in the rat brain to the region in the ventral surface of the rostral basal forebrain (3). Techniques such as *in situ* hybridization will therefore be necessary in the elucidation of the precise localization of the hDP receptor.

Comparison of the Human DP Receptor with Other Prostanoid Receptors—The hDP and mouse DP receptors are 73% identical at the amino acid level which increases to 85% in the TMDs. Amino acid sequence comparison of all members of the human prostanoid receptor family is shown in Fig. 6. In general, the prostanoid family shows 28% to 45% amino acid sequence identity between any two members. There are a total of 28 amino acid residues conserved in all prostanoid receptors, 8 of which are also conserved in all rhodopsin-like GPCRs. Of the 28 residues, only 6 of them occur outside of putative TMDs, and this includes the invariant and unique tripeptide, WCF, found in the second extracellular loop. The cysteine residue within the tripeptide forms a putative disulfide bridge with the conserved cysteine residue in the first extracellular loop. TMD VII is the most highly conserved TMD within the prostanoid receptor family, and it contains a conserved arginine residue, which has been postulated to act as the counterligand for the α-carboxyl moiety occurring in the prostanoid ligands (49). TMD VII also contains another invariant tripeptide, DPW, which includes the proline residue conserved in all GPCRs (44).

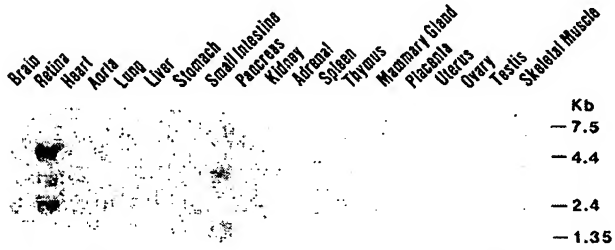

FIG. 5. **Northern blot analysis of human tissue RNA.** Poly(A)$^+$ RNA (3 μg) from 19 different human tissues, listed above each lane, was applied. Hybridization analysis was carried out using a ^{32}P-labeled hDP cDNA fragment as described under "Materials and Methods." The positions of the RNA markers on the gel are indicated.

Phylogenetic comparison of the prostanoid receptor family, shown in Fig. 7, reveals two main subfamilies, one comprising EP_1, TP, FP, and EP_3 and the other grouping EP_4, DP, IP, and EP_2. EP_1, TP, and FP are the most closely related within the first subfamily and have been shown to couple to an increase in $[Ca^{2+}]_i$. EP_3 receptors, which are less related within this subfamily, can couple to both elevation of $[Ca^{2+}]_i$ and a decrease in $[cAMP]_i$ (50). EP_2, DP, and IP are the most highly related receptors within the second subfamily of the prostanoid receptors and, together with EP_4, couple to elevation of $[cAMP]_i$. Interestingly, the mouse IP receptor has also been shown to couple to phosphatidylinositol metabolism when expressed in CHO cells (51), although the agonist concentration needed to generate IP_3 was approximately 3 orders of magnitude higher than that required to stimulate cAMP production. It remains to be established whether the subfamily of prostanoid receptors related to IP can all generate IP_3 upon activation and can, therefore, signal through a dual pathway. Moreover, given the

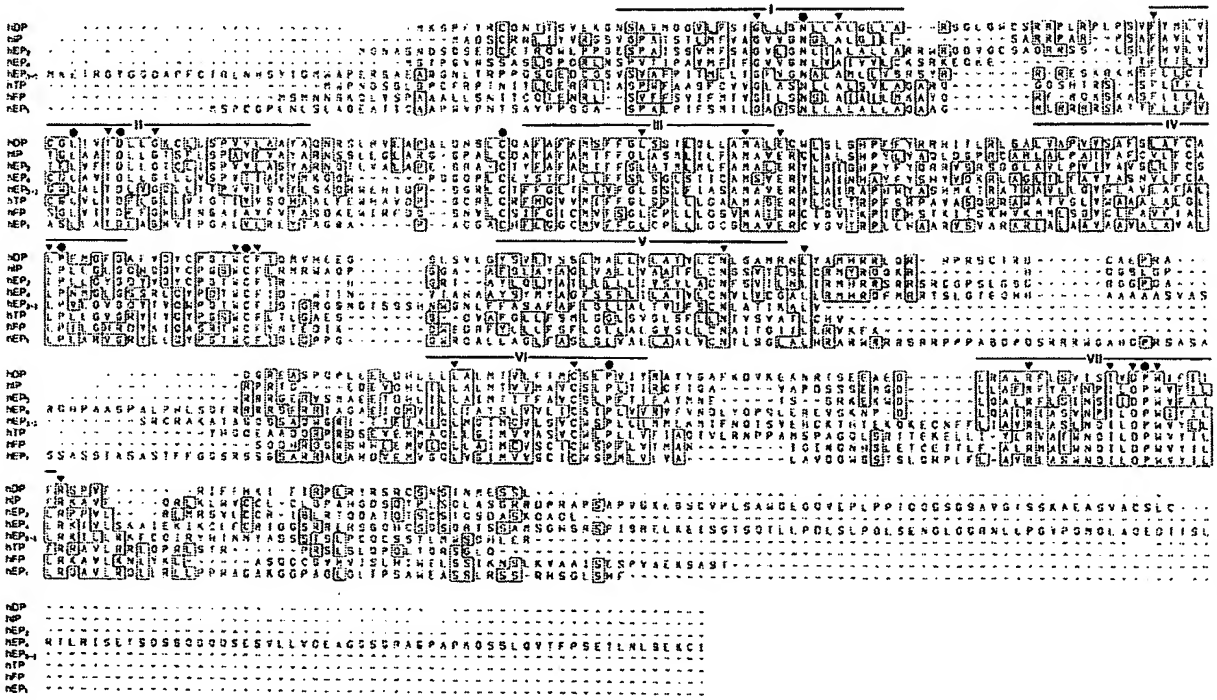

FIG. 6. **Amino acid sequence alignment of the human prostanoid receptor family.** The deduced amino acid sequences of the human DP, IP, EP_2, EP_4, EP_{3-1}, TP, FP, and EP_1 prostanoid receptors are shown aligned using GCG Wisconsin DNA software. Identical amino acids in at least three sequences are *boxed*. *Dashes* indicate gaps introduced in the sequences for alignment purposes. The TMDs are indicated by *overlines*. Conserved amino acids in all prostanoid receptors are indicated by a ▲, and those conserved in all rhodopsin-like GPCRs are indicated by a ●.

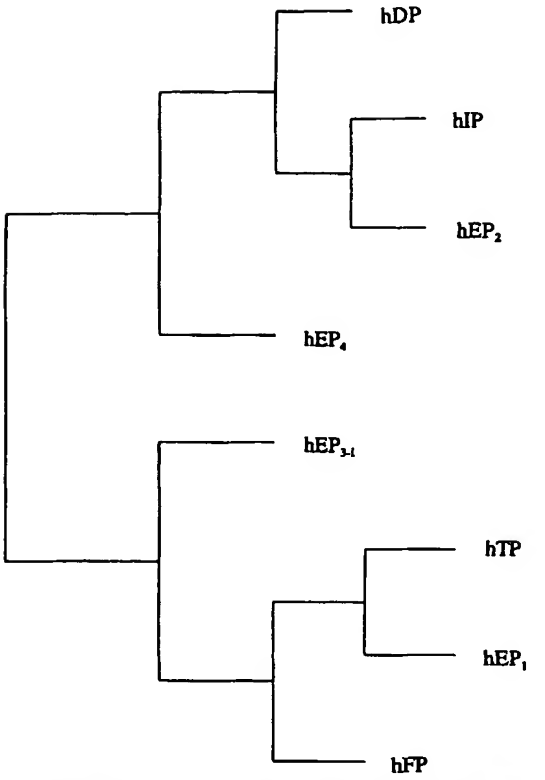

FIG. 7. **Phylogram of the human prostanoid receptor family.** Alignment of the deduced amino acid sequences of human prostanoid receptors (21, 22, 24, 27–29, 32) using the Kimura protein distance analysis (53) from the Wisconsin Sequence Analysis Package (Version 8.0). Branch lengths are proportional to calculated distances.

relatively high concentrations of agonist required to trigger IP_3 in this recombinant system, the physiological relevance of this signaling pathway remains to be determined.

In summary, we have cloned a cDNA for the hDP prostanoid receptor and have shown that this receptor is coupled to stimulation of intracellular cAMP production as a major signaling pathway. In addition, hDP has a discrete distribution as assessed by Northern blot analysis. The availability of the hDP cDNA will facilitate the elucidation of the ligand binding and signal transduction characteristics of this prostanoid receptor and will be essential in delineating the cellular distribution of hDP within tissues.

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